THREE-DIMENSIONAL IMAGE ACQUISITION SYSTEM FOR MULTI-SPERM TRACKING

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ABSTRACT

Sperm motility has been widely studied in two dimensions (2D) by analyzing their bidimensional trajectories when swimming near a flat surface. Under real conditions, the spermatozoid swims in a three-dimensional space before finding its target, the egg. The main restriction to track three-dimensionally these flagellated cells is their speed. Here we describe a novel method that allows the acquisition of data for three dimensional multi-tracking and analysis of free-swimming sperm trajectories. The system uses a piezo-electric device displacing a large focal-distance objective mounted on an inverted microscope (over its optical axis) to acquire 70 stacks of 60 images at a rate of 4,200 images of 512 x 512 pixels per second, over a depth of 100 μm. With this method whole 3D paths could be visualized during 1 s and measured for multiple sperm in the same field simultaneously for the first time. This new acquisition method extends the analytic possibilities from a restricted 2D situation to real 3D conditions.

Index Terms— Sperm Tracking, Three-Dimensional Analysis, Motility.

1. INTRODUCTION

Tracking sperm in 3D, as they naturally swim, has been an ambitious endeavor. The main obstacle to recording trajectories in 3D is the high speed with which these flagellated cells move. While the qualitative 3D helical movement of sperm had been observed long ago [1], Baba [2] compared quantitatively for the first time the speed and diameter of 2D sperm paths when focusing the microscope at two different levels (near a flat surface and 300 microns down the surface level). Later, Crenshaw [3] reported the first system capable of tracking 3D trajectories of single sea urchin spermatozoa for quantitative analysis. He adapted a technology to track in 3D low-speed single microorganisms keeping them focused at the centre of the microscope field that was developed by Berg [4], but which was not directly applicable to high-speed microorganism as spermatozoa. The system proposed by Crenshaw was composed of two video cameras that were attached to two microscopes perpendicular to one another (one camera giving the X, Y coordinates and the other one the X, Z coordinates). Focusing the microscopes to the centre of a volume, images were recorded and analyzed frame by frame to determine the screen coordinates. Qualitative 3D trajectories were presented in this work in which exposure to an attractant stimulated sperm swimming. Quantitative parameters such as speed, curvature and torsion were also presented as an example for a single sperm [5]. Moreover, important works described the mathematical theory of helical motion and chemotaxis with simulated microorganisms [6,7,8,9,10,11].

Here we present a new image acquisition approach for 3D multi-tracking of spermatozoa using a single camera and a single microscope. A piezoelectric device was mounted between the long-distance objective of the microscope and the turret. This device was excited with an amplified ramp signal to make it vibrate at up to 70 cycles per second, while acquiring synchronously at different focal planes (in a depth of 100 μm) the images of free-swimming spermatozoa at a rate of 4,200 images per second. This produced the necessary data for tracking their trajectories in three dimensions.

To prove the usefulness of the proposed system, we present comparative data of the mean speed and the average number of revolutions/s of a sea urchin spermatozoa population when swimming freely in a three-dimensional sea-water volume and when swimming circularly in two dimensions over the surface of a Petri dish.

2. SYSTEM CONFIGURATION

2.1. Biological preparation

Sperm were obtained 'dry' from the sea urchin *Strongylocentrotrus purpuratus* (Marinus Inc, Long Beach, CA, USA; Pamanes S. A. de C.V., Ensenada, Mexico) by intracoelomic injection of 0.5 M KCl and stored on ice. Artificial sea water (ASW) contained (mM): 430 NaCl, 10 KCl, 10 $CaCl_2$, 23 $MgCl_2$, 25 $MgSO_4$, 2 $NaHCO_3$, and 1 EDTA (pH 8.0, 950–1000mosM). The coverslips were

briefly immersed into a 0.1% (wt/vol) solution of polyHEMA in ethanol, hot-air blow-dried to rapidly evaporate the solvent, and mounted in supplied reusable chambers. Dry sperm were diluted 1/10 in ASW low Ca^{2+} (1mM) pH 7.0, thereafter a 2 µl aliquot was further diluted ~$2.5x10^5$ volumes in ASW and were transferred to an imaging chamber.

2.2. The equipment

A schematic representation of the system setup is presented in Figure 1. A piezoelectric device P-725 (Physik Instruments, MA, USA) was mounted between a 40x 0.55 NA long-working distance objective (Nikon Ph 2 DL) and the inverted microscope (Nikon Eclipse TE2000-U). This piezoelectric device was controlled by a servo-controller E-501 via a high current amplifier E-505 (Physik Instruments, MA, USA). The servo-controller was excited with a ramp signal from a 3311A function generator (Hewlett Packard, CA, USA). A synchronizing signal coming from the servo-controller was used for triggering the high speed camera Motion-Pro HS4 (Redlake, AZ, USA) with 4 Gigabyte RAM (for grabbing up to 2 seconds of 512 x 512 image-sequences; in our case 8,400 images). The microscope was mounted on a heavy metal plate which had an inflated inner tube resting on a heavy marble table to isolate the system from external vibrations. The imaging chamber containing the biological preparation was mounted in an external support inserted between the microscope objective and the light output to isolate it from the vibrations produced by the piezo-electric device. The temperature of the biological preparation was measured with a BAT-10R multipurpose +-0.1º rechargeable thermometer, using a BT-1 general purpose thermocouple probe (Physitemp Instruments, NJ, USA). System calibration was achieved using a calibrated glass slide (stage micrometer), with a 1 mm ruler having 10 µm divisions. Data acquisition and processing was achieved with a Pentium IV PC (1.8 Ghz).

2.3. Image acquisition of 3D stacks

The piezoelectric device was excited with a 70 Hz ramp waveform (in the open loop configuration) via the piezoelectric device (PD) high current amplifier. The open loop configuration of the PD means that no feedback signal is returned from the PD to the servo-controller. This configuration enabled a larger Z displacement (100 µm) of the PD while sacrificing the possibility of micro-positioning the PD to exact discrete points; nevertheless, the Z positions were obtained by linear interpolation after a calibration procedure described in section 2.4. The high speed video camera was synchronized to this movement to acquire 60 images for each 70 Hz cycle, employing an acquisition rate of 4,200 images per second (Fig. 2). This configuration warranted a Z resolution of 1.6 microns required for optimal 3D tracking of sperm, given that the mean size of their head is around 4 microns and that 1/60 s between stacks is largely sufficient to permit X,Y tracking of sperm since their X,Y displacement between stacks is around 3.3 microns for a mean speed of 200 microns/s. Sequences of 1.5 seconds (105 stacks) were recorded for each sampling condition using the camera's memory buffer and transferred via the USB-2 port to the computer as an .AVI compressed file.

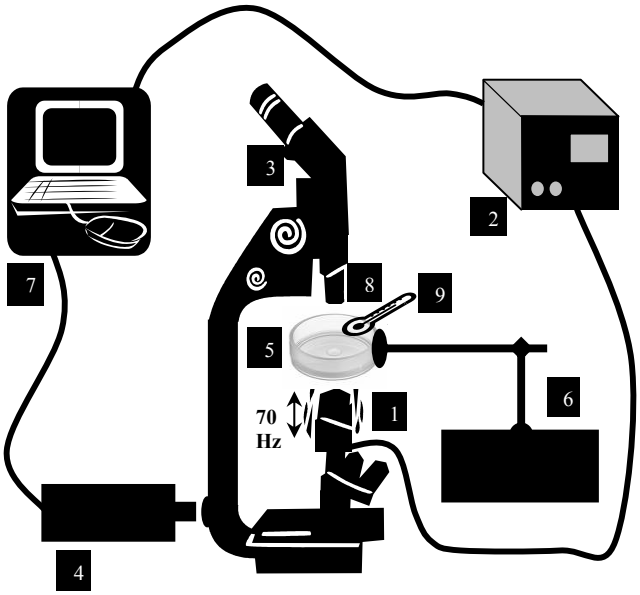


Fig. 1 Configuration of the 3D multi-sperm image acquisition system. 1- Piezoelectric device attached to the microscope long focal distance objective; 2- Servo-controller; 3- Inverted microscope; 4- High speed video camera; 5- Petri dish; 6- Isolated support for Petri dish; 7- Computer; 8- Light output; 9- Thermocouple probe (+-0.1º C).

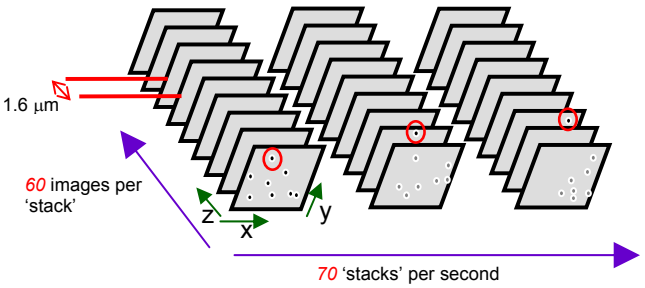


Fig. 2 High speed image stack acquisition for multi-sperm tracking. The circle surrounding the black point represents a single sperm moving over the 3D space of image stacks.

2.4. System calibration

For X,Y calibration of the system, the stage micrometer was immersed in the Petri dish filled with sea water to avoid calibration errors due to optical refraction of light. Since the PD was configured in open loop for a larger Z displacement, an external Z calibration was required. This was achieved

by measuring the maximal displacement provided by the piezoelectric device when applying the ramp 70 Hz signal. For this purpose, a fixed circular object was located at the centre of the field of observation while several image stacks were recorded at 4,200 images per second (at this time, the focus of the microscope is moving according to the ramp signal provided). Each stack contains a set of images of the fixed object while being maximally defocused when the ramp signal changes direction and in focus when its amplitude passes through its middle. The most defocused images were segmented with a fixed threshold corresponding roughly to the 'aura' of the object and the mean optical density within the circumference was measured at this point. Then, the piezoelectric device was turned off; at this point, the micrometrical knob of the microscope (1 μm resolution graved scale) was turned manually to find exactly the same defocused images of the fixed object (measuring the optical density), while the real displacement of the Z focal plane was deduced from the microscope's focus wheel. This measured the largest Z displacement between acquired stacks which accurately provided the corresponding calibration. This procedure was repeated for each sample of experimental conditions.

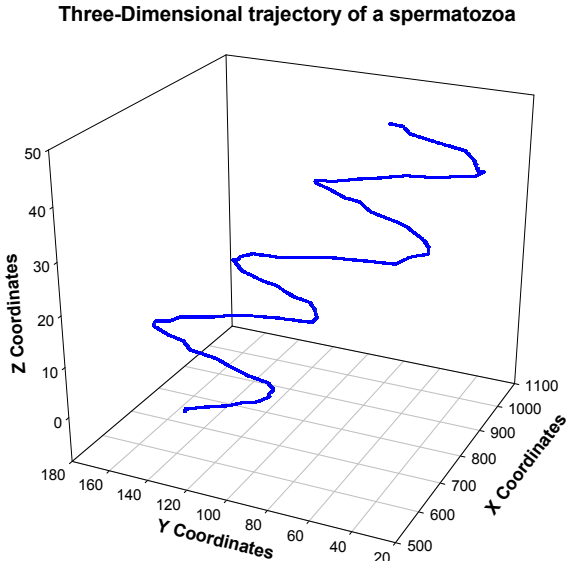
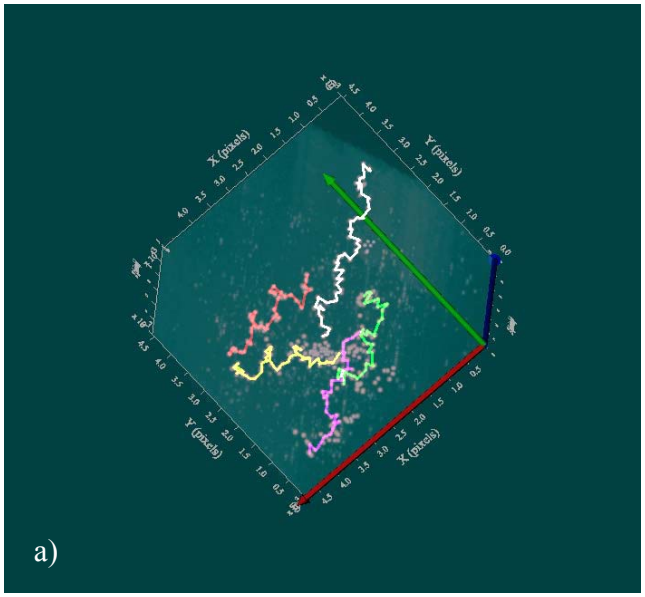


Fig. 3 Trajectory of a tracked single sea urchin spermatozoa swimming freely during 1 s, (units in microns).

2.5. 3D tracking of spermatozoa

Multiple high-performance 3D tracking algorithms have already been published such as Genovesio et al, [12]. Under our experimental conditions, sperm tracking was achieved manually as the main focus of the present work was the high speed acquisition of 3D data. The first 60 image stack was scanned to find 'in focus' sperm which were tagged. Since the X,Y,Z displacement of the sperm was small between stacks, it was simple to locate visually the same tagged spermatozoa within subsequent stacks. 3D coordinates of tracked sperm were recorded. Further development of automated video tracking procedures in our group is being achieved.



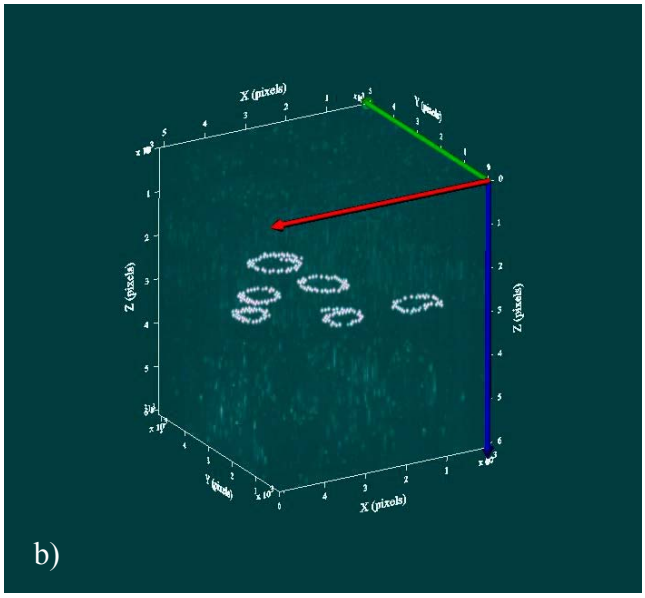


Fig. 4 Real reconstructed 3D scenes showing the trajectory of multiple tracked spermatozoa for a period of 1 s**.** a) Free-swimmers, b) Swimming sperm confined in 2D. Tracked sperm are coloured for visual purposes.

3. RESULTS

Figure 3 shows the helical trajectory of single tracked spermatozoa swimming freely for a period of 1 s. Figure 4 illustrates two real 3D reconstructed scenes (free-swimmers and confined sperm swimming in 2D) with several sea urchin sperm tracked in a sea-water volume in a Petri dish, both for a period of 1 s.

We have analyzed the speed of two groups of 9 spermatozoa swimming in the same volume at the same time. The first group swam near the flat surface of the Petri dish (describing circular trajectories), while the second group swam, freely in the sea water volume (describing helical pathways). The surface-confined group swam 21% ($\alpha < 0.05$) slower than the second group (one-factor ANOVA). Moreover, the average number of complete circular revolutions for the first group (1 revolution/s) was 3 fold fewer than the 3D free swimmers. Thus the trajectory curvature varies between these two populations of sperm.

4. CONCLUSIONS

We have presented a novel method that allows acquiring three-dimensional information for multiple free-swimming sperm simultaneously during 1 s. By measuring simple parameters as mean speed and number of completed circular revolutions in two different sperm groups, we show that the proximity to a nearby surface impacts the motile behaviour of sea urchin sperm. This may have important implications for currently published analyses of sperm swimming parameters and their dynamic regulation during processes such as chemotaxis, which have been nearly universally obtained with confined sperm swimming in 2D (e.g. [13]). Such studies must be viewed with precaution given our current results which show that sperm trajectories in (more physiologically relevant) 3D are significantly at variance with those of swimming sperm confined in 2D. Further studies are ongoing to better characterize these changes.

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